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Pathfinding of corticothalamic axons relies on a rendezvous with thalamic projections

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Summary

Major outputs of the neocortex are conveyed by corticothalamic axons (CTA), which form reciprocal connections with thalamocortical axons, and corticosubcerebral axons (CSA) headed to more caudal parts of the nervous system. Previous findings establish that transcriptional programs define cortical neurons identity and suggest that CTA and thalamic axons may guide each other, but the mechanisms governing CTA versus CSA pathfinding remain elusive. Here, we show that thalamocortical axons are required to guide pioneer CTA away from a default CSA-like trajectory. This process relies on a hold in the progression of cortical axons, or waiting period, during which thalamic projections navigate towards cortical axons. At the molecular level, Sema3E/PlexinD1 signaling in pioneer cortical neurons mediates a "waiting signal" required to orchestrate the mandatory meeting with reciprocal thalamic axons. Our study reveals that temporal control of axonal progression contributes to spatial pathfinding of cortical projections and opens novel perspectives on brain wiring.

Keywords

corticothalamic; thalamocortical; axon guidance; reciprocal connections; waiting period; Sema3E; PlexinD1; handshake

Introduction

Reciprocal connections between brain structures provide critical feedback and feedforward loops in major neural circuits. However, how they are established during development remains largely to be determined. Reciprocal connections between the thalamus and the neocortex are formed by thalamocortical (TCA) and corticothalamic (CTA) axons that convey sensory and motor information essential for cortical functioning. TCA and CTA contribute to the internal capsule, a large axonal highway navigating through the basal ganglia or subpallium, which also comprises output corticosubcerebral axons (CSA) en route towards the cerebral peduncle and pyramidal tract (Auladell et al., 2000; Price et al., 2006). The internal capsule is thus a major gateway to and from the neocortex. Studies over

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the past decades have focused onto the mechanisms governing its development, particularly on the role of transcription factors in CTA and CSA specification and on the function of subpallial guidepost cells in TCA pathfinding.

CTA and CSA are generated by corticofugal pyramidal neurons located in distinct layers: subplate and deep layer VI generate pioneer CTA neurons (pCTA), layer VI contains CTA neurons that grow in a second step, and layer V produces CSA neurons (McConnell et al., 1989, 1994; De Carlos and O'Leary, 1992; Auladell et al., 2000; Del Río et al., 2000; Molyneaux et al., 2007). Recent experiments have demonstrated that this laminar specificity is controlled by distinct transcriptional programs which define the identity of cortical pyramidal neurons, including their axonal trajectory (Arlotta et al., 2005; Chen et al., 2008; Kwan et al., 2008; Lai et al., 2008; Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011; Shim et al., 2012). In parallel, the subpallium has been shown to constitute an essential intermediate target for axons of the internal capsule (Métin and Godement, 1996). During embryogenesis, pCTA and TCA reach the subpallium and progress in opposite directions towards their reciprocal targets while staying in close vicinity (Molnár et al., 1998; Auladell et al., 2000; Bellion et al., 2003; Jacobs et al., 2007; Grant et al., 2012). Analyses of mutant mice have shown that the subpallium controls the navigation of CTA, CSA and TCA by secreting guidance factors and generating guidepost cells (Métin et al., 1997; Richards et al., 1997; Braisted et al., 1999; Tuttle et al., 1999; Hevner et al., 2002; Tissir et al., 2005; Uemura et al., 2007; Zhou et al., 2008; Magnani et al., 2010; Molnár et al., 2012).

The subpallium comprises the lateral ganglionic eminence (LGE) and the medial ganglionic eminence (MGE), which participate in the guidance of distinct axonal populations. In particular, LGE-derived corridor neurons have been shown to guide TCA along their route towards the neocortex (López-Bendito et al., 2006; Bielle et al., 2011a, 2011b). These guidepost neurons migrate from the LGE into the MGE, and form a permissive corridor for TCA deep to the globus pallidus (GP). In addition, the MGE-derived GP has been associated with CSA pathfinding, as CSA navigation is specifically affected in *Nkx2.1* mutant mice that impair MGE development (Marín et al., 2002). In contrast, the structures or mechanisms governing pCTA pathfinding have remained elusive. The close vicinity of CTA and TCA together with analyses of mutant mice affecting one of the two axonal populations have suggested that these reciprocal projections might generally guide each other (Molnár and Blakemore, 1995; Molnár et al., 1998; Hevner et al., 2002; Jones et al., 2002; López-Bendito et al., 2002; Wu et al., 2010; Chen et al., 2012, 2012). However, this hypothesis has remained controversial (Bagnard et al., 2001; Torii and Levitt, 2005), particularly since TCA and cortical axons collapse each other *in vitro* (Bagnard et al., 2001).

Here, we investigated the mechanisms governing the pathfinding of somatosensory pCTA. We found that pCTA and CSA follow distinct trajectories within the subpallium: pCTA navigate together with TCA in the permissive corridor, whereas CSA grow in the GP before joining the cerebral peduncle. Using a combination of *in vivo* genetic ablation of the thalamus and *ex vivo* experiments, we demonstrated that TCA are required to guide pCTA into the corridor. In absence of TCA, pCTA follow a default CSA-like trajectory. This guidance function of TCA relies on a pause, or waiting period, in the progression of corticofugal axons given that pCTA reached the lateral subpallium at least a day before TCA. At the molecular level, PlexinD1/Semaphorin3E signaling is required to prevent premature subpallial progression of pCTA before TCA have reached the proper position, thereby ensuring that pCTA follow their normal trajectory. Taken together, these results show that a waiting period controls the pathfinding of pCTA, by allowing their interaction with incoming reciprocal TCA. Our study reveals how temporal regulation of axonal

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progression regulates the pathfinding of pCTA and opens novel perspectives on the role of timing in the formation of brain circuits.

Results

pCTA and CSA follow distinct trajectories within the subpallium

To investigate the mechanisms controlling pCTA pathfinding, we first examined the precise localization of pCTA and CSA in the subpallium using axonal tracing experiments with carbocyanine dyes (Figures 1 and S1). Axonal paths were characterized at E17.5, when TCA have reached the neocortical subplate, pCTA the thalamus and CSA the cerebral peduncle (McConnell et al., 1989, 1994; Molnár et al., 1998; Auladell et al., 2000; Jacobs et al., 2007). Hence, DiI injections in the presumptive somatosensory cortex and DiA injections in the thalamus labeled axons exiting and entering each of these structures (Figures 1A-1B). While TCA and pCTA co-stained by DiA and DiI formed a compact tract in the corridor (López-Bendito et al., 2006), CSA only labeled with DiI were positioned in the deep GP visualized by Nkx2.1 immunostaining, and joined the cerebral peduncle (n=7) (Figures 1A-1B and 1E). By performing additional tracing experiments from the thalamus and cerebral peduncle as well as from the internal capsule, we similarly found that pCTA and CSA have segregated trajectories in the corridor and GP, respectively (Figures 1C and S1). We furthermore examined globally the trajectory of cortical axons by taking advantage of the cortex-specific $Emx1^{Cre}$ mouse line (Gorski et al., 2002) backcrossed to the reporter line Tau^{GFP} (Bielle et al., 2005). Using a 45° plane of section which encompasses the entire projection, we consistently observed that: i) subsets of cortical axons navigate in the corridor with TCA, labeled by 2H3 neurofilament immunostaining; ii) others form a less compact bundle in the Nkx2.1-expressing GP and extend into the cerebral peduncle (n=5) (Figures 1D and 1D'). Taken together, our results show that somatosensory pCTA and CSA have distinct trajectories within the subpallium, with pCTA navigating along the same route as TCA in the corridor and CSA growing in the deep GP (Figure 1F).

pCTA switch to a CSA-like trajectory in the absence of thalamic axons *in vivo*

pCTA navigate within the corridor, raising the possibility that corridor neurons may directly guide pCTA or that TCA may orient pCTA pathfinding into the corridor. To discriminate between these possibilities, we first performed a genetic ablation of the thalamus to assess its long-distance impact on the pathfinding of pCTA within the subpallium. To this aim, we backcrossed $Wnt3a^{DTA}$ mice (Yoshida et al., 2006) that conditionally express the subunit A of the diphtheria toxin (DTA) in the cortical hem and thalamus (Yoshida et al., 2006; Louvi et al., 2007) with $Brn4^{Cre}$ mice that drive the expression of Cre recombinase in the embryonic neuroepithelium albeit not efficiently in the cortical hem (Ahn et al., 2001; Heydemann et al., 2001). In $Brn4^{Cre};Wnt3a^{DTA}$ embryos, complete thalamus ablation was detected by tissue loss and lack of thalamic molecular markers (Figures 2A, 2B, S2 and data not shown), with a subsequent absence of TCA (Figures 2C and 2D). Using specific markers, we found that the patterning of the prethalamus and the subpallium (*Ebf1*, Islet1, Nkx2.1, Ctip2, Foxp2) as well as cortical lamination (Calretinin, Tbr1, Ctip2, ZFPM2), were not affected in thalamusablated embryos (n=8) (Figures 2E-2J, S2 and data not shown). We next examined the subpallial trajectory of pCTA in the absence of TCA, using DiI labeling in the presumptive somatosensory neocortex and L1 immunostaining, which labels both cortical and thalamic axons (Figures 3A-3F and S3). We observed that corticofugal axons grew normally into the subpallium at E14.5 (n=6) (Figures 3A, 3B and S3), indicating that this event is independent of incoming TCA. However, in E16.5 and E18.5 thalamus-ablated embryos, all the labeled corticofugal axons passed through the Nkx2.1-expressing GP and reached the cerebral peduncle (nE16.5=5 and nE18.5=4) (Figures 3D, 3D', 3F and S3). This is in sharp contrast with the control situation in which pCTA and TCA navigate in the

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corridor and CSA in the GP (Figures 3C, 3C', 3E and S3). Our observations indicated that in thalamus-ablated embryos, pCTA either do not grow into the subpallium or adopt an abnormal CSA-like trajectory. To discriminate between these possibilities, we back-labeled cortical neurons that had extended axons in the corridor or GP at E17.5 and E18.5 (Figures 3G, 3H and data not shown). In both controls and thalamus-ablated embryos, we labeled a large majority of neurons in the subplate and layer VI, as well as in layer V ($n_{E17.5}$=4 and $n_{E18.5}$=4) (Figures 3G, 3H and data not shown). Taken together, these observations showed that, in thalamus-ablated embryos, subplate and deep layer VI pCTA are misguided along an alternative CSA-like trajectory (Figures 3I and 3J) in spite of their CTA-specific molecular identity (Figures 3H and S2). Our results thus reveal that corridor cells and subpallial structures are not sufficient to guide pCTA and that the ablation of the thalamus has a distal impact on the trajectory of pCTA in the subpallium.

TCA are necessary to guide pCTA into the corridor

Since our *in vivo* analysis strongly suggested that TCA might guide pCTA, we set up an *ex vivo* assay to directly test this hypothesis. We took advantage of the fact that TCA grow along a 45° angle trajectory and are thus present in E13.5 caudal coronal slices but not yet present in E13.5 intermediate coronal slices (Figure 4A and data not shown). In such TCA-free intermediate slices, we grafted cortical explants from transgenic embryos ubiquitously expressing the green fluorescent protein (GFP) and found that *Gfp*-expressing corticofugal axons did not enter the corridor but massively grew into the GP (n=60/60) (Figure 4B), as in thalamus-ablated embryos. Using DiI retrograde labeling of GFP-positive axons that had extended into the GP (n=4) (Figure 4C), we found that these axons are mainly pCTA as they are generated by subplate and deep layer VI neurons (Figure 4D). Thus, *ex vivo* as *in vivo*, pCTA adopt an alternate CSA-like trajectory in the absence of TCA. To exclude that this behavior was due to *ex vivo* culture conditions, we performed 45° angle slices that contained TCA and found that GFP-positive cortical axons grew normally both into the corridor to the thalamus, and into the GP to the cerebral peduncle (n=12/12) (Figures 4E and 4F).

To next determine whether TCA are essential to guide pCTA into the corridor, we performed grafts of thalamic explants harvested from *tdTomato*-expressing embryos or from control embryos and labeled with DiI (Figure 4G). When thalamic explants were grafted at the ventral tip of the corridor and generated TCA, we found GFP-positive pCTA growing in the Islet1-positive corridor, in vicinity of TCA (n=29/37) (Figures 4I and 4J). This is in striking contrast to control incisions in which pCTA did not grow in the corridor and directly entered the GP (n=37/37) (Figure 4H). This difference in cortical axonal trajectory was very robust since we found that pCTA enter the Islet1-expressing corridor in 78% of slices in which thalamic explants were grafted and extended TCA (n=29/37) (Figure 4L). Finally, when thalamic explants were grafted in the lateral cortex, they did not induce a deviation of pCTA trajectory (n=10) (data not shown). This observation indicates that thalamic explants do not produce a diffusible long-range signal, but rather act on cortical axons pathfinding via a local activity of thalamic axons. Consistent with this finding, high magnification of rescue experiments showed that TCA and CTA, although not tightly fasciculated, grow in close vicinity within the corridor (Figure 4K). Overall, our data shows that the presence of TCA inside the corridor is both necessary to guide pCTA and sufficient to prevent them from undertaking an alternative CSA-like trajectory.

Waiting period of cortical axons coordinates their progression with TCA

Our results raised the intriguing question of how pCTA and TCA progression is coordinated to ensure the proper pathfinding of pCTA. To determine the underlying mechanisms, we re-examined the precise timing of cortical and thalamic axons progression by implantation of DiI and DiA crystals in the presumptive somatosensory cortex or thalamus, respectively

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(Figure 5). Consistent with previous studies (McConnell et al., 1989, 1994; Métin and Godement, 1996; Molnár et al., 1998; Auladell et al., 2000; Bellion et al., 2003; Jacobs et al., 2007), we found that pioneer corticofugal axons exited the cortex and, between E13.5 and E14.5, paused in the adjacent lateral part of the subpallium, or lateral striatum (Figures 5A and 5B). After this waiting period, corticofugal axons progressed into the subpallium and DiI back-labeling from the internal capsule indicated that subplate pCTA progressed by E15, followed by layer V CSA at E17.5 (Figure S4). In contrast to the waiting behavior of cortical axons, between E13.5 and E14.5, TCA progressed through the subpallium to reach the lateral striatum (Figures 5C-5F). Thus pCTA enter the subpallium at least a day before TCA, and halt their progression during a waiting period which allows TCA to reach their location. Since TCA guide pCTA, these findings reveal a major role for the waiting period in pioneer cortical axons pathfinding.

To determine if this waiting period is regulated by local signals, we performed *ex vivo* experiments in coronal slices and found that *Gfp*-expressing cortical axons pause for a day similarly as they do *in vivo* (Figures 5G-5I). The waiting period is thus likely controlled by dynamic changes in either corticofugal axons or subpallial cells. Dynamic changes in cortical neurons between E13.5 and E15.5 were revealed by a refinement of their molecular identity as well as by a modification of their axon guidance properties. Indeed, we found that the expression of the transcription factors Tbr1 and Ctip2, which are essential for defining the identity and pathfinding of CTA and CSA, respectively (Hevner et al., 2002; Bedogni et al., 2010; Han et al., 2011; McKenna et al., 2011), are initially co-expressed at high levels at E13.5 and become progressively restricted to each neuronal cell type (Figure S4). More importantly, when we performed heterochronic grafts of E13.5 to E15.5 cortical explants into E13.5 wild type host slices, we observed that cortical axons from older explants do not pause when confronted with a younger subpallium (n=10/11)(Figures 5J-K' and data not shown). Taken together, these results indicate that the waiting period of pioneer corticofugal axons, which is required for their "encounter" with TCA, is regulated by temporal modifications in cortical neurons properties.

PlexinD1 and Sema3E are candidate factors for the waiting period

To characterize the molecular cues controlling this mandatory waiting period, we searched for candidate receptors transiently expressed by corticofugal neurons. We particularly examined Plexin and Neuropilin receptors, as they have been involved in the regulation of waiting periods in other systems (Huettl et al., 2011). Using this strategy, we focused on PlexinD1, a member of the plexin family that has been shown to bind directly to its ligands, including the secreted semaphorin 3E (Sema3E) (Torres-Vázquez et al., 2004; Gu et al., 2005). *PlexinD1* expression was detected from E15.5 in the striatum and the lateral cortex (including piriform and insular cortex) but not in the dorsal neocortex (Chauvet et al., 2007). In addition, while *PlexinD1* and *Sema3E* inactivation have been reported to perturb the late development of some *PlexinD1*-expressing axons (Chauvet et al., 2007), their early expression or phenotype had not been described. We found that *PlexinD1* is transiently highly expressed in the preplate and subplate during the waiting period at E13.5 and E14.5 (Figures 6A-6D) and PlexinD1 protein is detected on corticofugal neurons labeled by Tbr1 and Ctip2 co-immunostainings (Figures 6E). From E15.5 onwards, *PlexinD1* expression is maintained in the lateral cortex while it is downregulated in the neocortex (Figures 6C and 6D). Consistent with this observation, PlexinD1 protein could not be detected on corticofugal axons after E15.5 (Figure 6F). Since PlexinD1 was shown to mediate either repulsion alone or attraction when co-expressed with Neuropilin1 and VEGFR2 (Chauvet et al., 2007; Bellon et al., 2010), we examined the expression patterns of these putative co-receptors. We found that neither Neuropilin1 nor VEGFR2 is present on corticofugal axons during the waiting period (Figures 6G and 6H), suggesting that PlexinD1 could mediate a

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repulsive or growth-inhibiting signal in early corticofugal axons. Consistent with this finding, we found that Sema3E is expressed in the radial glia of the striatum and in the GP (Figure 6I) and could thus act on cortical axons as they enter the subpallium. To characterize the activity of Sema3E on cortical axons, we performed collapse assays on dissociated neurons prepared at E13.5 or E15.5 from either the dorsal neocortex, which down-regulates *PlexinD1* expression, or from the lateral cortex, which maintains *PlexinD1* expression (Figure 6J). While lateral cortical neurons collapsed at E13.5 and E15.5 when exposed to Sema3E, neocortical neurons massively collapsed at E13.5 but showed no significant response at E15.5 (Figures 6K-6N). These results indicate that PlexinD1/Sema3E signaling mediated by transient expression of *PlexinD1* is a prime candidate for the regulation of the waiting period of corticofugal axons.

Sema3E/PlexinD1 signaling controls the waiting period and pCTA pathfinding

To investigate the role of Sema3E/PlexinD1 signaling in the waiting period, we examined both $Sema3E^{-/-}$ mutant embryos (Gu et al., 2005) and $Emx1^{Cre};PlexinD1^{\Delta/flox}$ mouse embryos (Gorski et al., 2002; Pecho-Vrieseling et al., 2009; Zhang et al., 2009), which carry a cortex-specific deletion of the *PlexinD1* gene (Figure S5). We first checked using DiI labeling, immunohistochemistry and *in situ* hybridization, that the growth and pathfinding of TCA, the patterning of the subpallium (Ctip2, Nkx2.1, *Ebf1* and Islet1) as well as the layering and maturation of the neocortex (Calretinin, Ctip2, Foxp2, Tbr1, ZFPM2) were not affected either by *Sema3E* or *PlexinD1* cortical inactivation (n=9 for each genotype) (Figure S6 and data not shown). We next examined the early pathfinding of corticofugal axons using DiI injections in the presumptive somatosensory neocortex (Figures 7A-7D”). In contrast to control embryos in which corticofugal axons paused at E13.5 and E14.5 in the lateral striatum (Figures 5A-5F, 7A and 7A’), corticofugal axons were detected as early as E14.5 in the GP and cerebral peduncle of both $Sema3E^{-/-}$ and $Emx1^{Cre};PlexinD1^{\Delta/flox}$ embryos (n=10 for $Sema3E^{-/-}$ and n=6 for $Emx1^{Cre};PlexinD1^{\Delta/flox}$) (Figures 7C-7D”). Using immunohistochemistry and DiI back-labeling from the GP, we found that these axons belonged to neurons located in the subplate and layer VI which expressed the corticothalamic-specific gene ZFPM2 (n=3 for $Sema3E^{-/-}$ and n=6 for $Emx1^{Cre};PlexinD1^{\Delta/flox}$) (Figures 7I and 7J). Thus in the absence of Sema3E/PlexinD1 signaling, the waiting period is waived, pCTA enter prematurely into the subpallium before TCA arrival and follow a CSA-like trajectory.

To further determine whether these early defects in pioneer corticofugal axons impact on the trajectory of CTA, we focused on neonates (Figure 8) since *PlexinD1* starts to be expressed perinatally in layer V neurons (Arlotta et al., 2005; Chauvet et al., 2007), where it might play additional roles. Using L1 immunostaining (n=3 for each genotype) and DiI cortical tracing (n=3 for each genotype), which label at this stage not only cortical but also thalamic axons, we observed no major tracts defects in mutant mice (Figures 8A-8F). These findings indicate that the internal capsule is not severely perturbed, which is consistent with our early analysis showing normal TCA pathfinding, but does not exclude that CTA may present pathfinding defects. To directly test whether some CTA follow an abnormal CSA-like trajectory in mutant neonates, we performed focal insertion of DiI crystals in the cerebral peduncle to specifically label cortical neurons that had extended their axons into this tract (n=9 for controls, n=7 for $Sema3E^{-/-}$ and n=10 for $Emx1^{Cre};PlexinD1^{\Delta/flox}$). Accuracy of crystal placement and specificity of cerebral peduncle labeling were systematically assessed after DiI diffusion (Figure S7). As expected, cerebral peduncle tracing back-labeled layer V neurons in both control and mutant mice (Figures 8G-8L). However, cerebral peduncle tracing additionally stained scattered neurons located in the subplate and layer VI, which expressed the CTA-specific marker ZFPM2, in both *Sema3E* and cortical-specific *PlexinD1* mutant mice (Figures 8G-8L). These results indicate that some CTA are misrouted along a

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CSA-like trajectory in mutant neonates. Taken together, our results reveal that Sema3E/PlexinD1 signaling regulates the waiting period and contributes to the guidance of pCTA by TCA.

Discussion

Our study shows that the waiting period of corticofugal axons contributes to the establishment of reciprocal connections between the thalamus and the cerebral cortex. This waiting period enables the arrival of reciprocal TCA, which are necessary to guide pCTA. Indeed, in the absence of TCA, cortical neurons mature normally but pCTA follow an alternative trajectory and the presence of TCA is sufficient to rescue this abnormal pathfinding. At the molecular level, transient Sema3E/PlexinD1 signaling in cortical neurons regulates this waiting period and thereby the pathfinding of pCTA. Taken together, our study reveals the role of axonal interactions in the formation of reciprocal projections as well as the importance of temporal checkpoints in the establishment of neural circuits.

Thalamic axons guide pioneer corticothalamic projections

From the early observation that TCA grow in the cortex along a scaffold of subplate axons, Molnár and Blackmore proposed the handshake hypothesis (Molnár and Blakemore, 1995). Reciprocal TCA and CTA appeared closely intermingled throughout the internal capsule (Molnár et al., 1998), raising the possibility that these two sets of axons guide each other. Several analyses of mutants affecting TCA pathfinding, including *Gbx2* and *Mash1* mutants (Tuttle et al., 1999; Hevner et al., 2002), showed defects in corticofugal tracts, thereby bringing support to the handshake hypothesis. Furthermore, it has been recently reported that mutants perturbing axonal fasciculation in the internal capsule generally affect corticofugal pathfinding (Wu et al., 2010). However, these analyses remain inconclusive as the observed defects could be due to subpallial abnormalities. In addition, *in vitro* studies revealed that thalamic and cortical axons repel each other (Bagnard et al., 2001). Since the navigation of TCA through the subpallium is known to be independent of CTA and relies on the presence of guidepost corridor neurons (López-Bendito et al., 2006), it has been proposed that corticofugal axons might be directly guided by subpallial cues. To directly investigate the mechanisms governing CTA navigation, we have focused on pioneer axons of the somatosensory cortex and revealed that pCTA and CSA have distinct trajectories in the subpallium: they grow predominantly through the corridor and the GP, respectively. Phenotypic analysis of $Brn4^{Cre};Wnt3a^{DTA}$ embryos showed that in absence of the thalamus, pCTA adopt a CSA-like trajectory at a long distance from the thalamus, within the subpallium. These observations revealed that subpallial guideposts, such as corridor cells are not sufficient to guide pCTA. They furthermore suggested that either the thalamus or thalamic axons guide pCTA. Using an *ex vivo* slice assay, we have consistently found that thalamic explants do not attract cortical axons when grafted in the lateral cortex (data not shown) or when confronted with cortical explants (data not shown). Similarly, when grafted thalamic explants did not extend axons into the corridor of host slices, they were unable to rescue the trajectory of cortical axons (data not shown). Thus, our findings are not supportive of a long-range trophic or chemotactic activity of the thalamus on pCTA trajectory. They instead indicate that TCA within the corridor are required to open a corticothalamic path. At cellular resolution, we found that TCA and pCTA navigate closely although do not tightly fasciculate. These results are consistent with the *in vitro* observations that TCA may not act as an axonal substrate for pCTA (Bagnard et al., 2001; Torii and Levitt, 2005). They rather indicate that either TCA induce a modification in the local subpallial environment that in turn acts on pCTA, or TCA provide a track for cortical axons, similarly to what has been described during the formation of peripheral motor and sensory nerves (Huettl et al., 2011; Wang et al., 2011). While further experiments will be required to

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investigate these issues, our findings are consistent with apparently previous contradictory studies (Molnár and Blakemore, 1995; Bagnard et al., 2001; Torii and Levitt, 2005; Chen et al., 2012). Remarkably, our work reveals that the formation of reciprocal connections between the thalamus and cortex relies on a timing of sequential events: the migration of conserved subpallial guidepost cells defines the trajectory of TCA (López-Bendito et al., 2006; Bielle et al., 2011a, 2011b), which in turn guide reciprocal pCTA. This later process ensures a developmental robustness in the establishment of reciprocal projections between the thalamus and cortex, even in cases of abnormal pathfinding of TCA (Tuttle et al., 1999; Hevner et al., 2002). Our findings furthermore open the intriguing possibility that such guidance mechanism may more generally govern the formation of reciprocal connections in the brain.

Waiting period is regulated by PlexinD1/Sema3E signaling

Studies over the past decades have revealed the central role of families of guidance cues in the spatial control of neural circuits formation. In particular, PlexinD1, an atypical member of the Plexin superfamily that directly binds to Sema3E, controls axonal pathfinding (Chauvet et al., 2007; Bellon et al., 2010) and synaptogenesis (Pecho-Vrieseling et al., 2009; Ding et al., 2011) in major neuronal networks. In this study, we show that the dynamic changes in Sema3E/PlexinD1 signaling in cortical neurons controls their waiting period (Figures 6 and 7), without apparently affecting their neuronal identity (Figure S6 and data not shown). Indeed, PlexinD1 signaling in cortical axons induces a growth inhibition effect in response to Sema3E, which is expressed by the radial glia of the striatum and by the GP. In addition, the full inactivation of *Sema3E* or the cortex-specific inactivation of *PlexinD1* disrupted the waiting period. The premature growth of cortical axons in both mutants occurs while TCA are still crossing the subpallium (Figure S6), contributing to an abnormal trajectory of pCTA along a CSA-like trajectory. This difference in trajectory is not due to a change in *Sema3E* activity in the GP, since a similar pathfinding defect is observed in thalamus-ablated embryos in which *PlexinD1* and *Sema3E* are normally expressed (data not shown). Finally, these early defects in pCTA pathfinding impact on the axonal trajectory at birth since at least some CTA still follow a CSA-like trajectory. Taken together, these results indicate that PlexinD1/Sema3E signaling regulates the spatial pathfinding of pioneer cortical axons by modulating the timing of their progression. Our study thus reveals that the temporal regulation of a guidance cue receptor controls a major checkpoint and thereby participates to the pathfinding of reciprocal projections between the thalamus and the neocortex.

Waiting period is essential for pioneer corticothalamic axons pathfinding

Assembly of neural circuits requires a precise spatial organization as well as a specific timing. While the spatial control of axon guidance and network formation has been well described, much less is known about the temporal regulation. Waiting periods of growing axons have been observed in several systems (Tosney and Landmesser, 1985; Sharma et al., 1994; Wang and Scott, 2000; Bloom et al., 2007) and have been suggested to let time for the maturation of axonal targets. However, the exact functions and regulatory mechanisms of waiting periods are largely uncharacterized. Here, we report that pCTA waiting period participates to two related but distinct processes. On the one hand, it allows TCA to reach the lateral subpallium, a mandatory event for the correct pathfinding of pCTA. On the other hand, it enables corticofugal neurons to complete their final maturation and specifically express transcription factors that are required for CTA and CSA pathfinding, such as Tbr1 and Ctip2 (Han et al., 2011; McKenna et al., 2011). Indeed, Ctip2 and Tbr1 are initially co-expressed in waiting cortical neurons and become progressively downregulated in pCTA and CSA, respectively. This transcriptional maturation occurs independently of TCA (Figure 2J and S2) as well as *in vitro* in dissociated cortical neurons (data not shown), which is

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consistent with a cortexintrinsic program. High levels of Ctip2 or Tbr1 are likely important to govern the expression of intrinsic axonal properties essential for the guidance of distinct corticofugal axons (Han et al., 2011; McKenna et al., 2011). Consistently, in *Tbr1* mutant, all corticofugal axons express high levels of Ctip2, adopt a layer V identity and all follow a CSA trajectory even in the presence of TCA (Hevner et al., 2002; Bedogni et al., 2010). In this context, our analysis intriguingly reveals that pCTA have the capacity to follow a default CSA-like trajectory in the absence of TCA, but also have a specific property to respond to the presence of TCA. The waiting period is thus a central temporal checkpoint as it orchestrates the timing of two major events: the intrinsic maturation of cortical neurons, including the acquisition of specific axonal properties in CTA, and the extrinsic arrival of reciprocal TCA. Our study reveals the functional relevance of timing in the navigation of a major reciprocal projection of the mammalian brain and provides novel insights on the function of waiting periods in the precise wiring of neural networks.

Experimental Procedures

Mouse Lines

The different mouse lines used in this study are described in detail in the supplementary information. The day of vaginal plug was considered as embryonic day (E) 0.5. Animals were kept under French and EU regulation.

In Situ Hybridization, Immunohistochemistry and Axonal Tracing

For *in situ* hybridization, mouse brains were fixed overnight in 4% paraformaldehyde in PBS (PFA) at 4°C. 100 μm free-floating vibratome sections were hybridized as described before (López-Bendito et al., 2006) with the following digoxigenin-labeled probes: *Sema3E* and *PlexinD1* (Chauvet et al., 2007), *Ebf1* (Garel et al., 1997). For axonal tracing, embryonic brains were fixed at least overnight at 4°C and cultured slices for 30 min in 4% PFA at RT. Small crystals of DiI (1,1′-dioctadecyl 3,3,3′,3′-tetramethylindocarbo-cyanine perchlorate; Molecular Probes) or DiA (4-4-dihexadecyl aminostyryl N-methyl-pyridinium iodide; Molecular Probes) were inserted into the thalamus, the cerebral cortex, the internal capsule and the GP or the cerebral peduncle after hemi-dissection of the brains or into cultured slices and let diffuse at 37°C (from two days up to a month). Status of dye diffusion was assessed by whole-brain or whole-explant examination under a fluorescent binocular set-up (Leica MZ16 F). Subsequently, brains were cut into 100 μm-thick vibratome sections and tracing specificity was systematically checked after diffusion on serial sections adjacent to the site of crystal insertion. Some sections were processed for immunohistochemistry as described below.

For immunohistochemistry, cultured slices/explants and embryos were fixed in 4% PFA at 4°C for 30min and for 4–12 hours, respectively. Immunohistochemistry was performed on culture slices or 80 μm–100 μm free-floating vibratome sections. The slices were incubated 1h at RT in a blocking solution adapted to the primary antibody used (see supplementary information), incubated in the same blocking solution with primary antibodies overnight at 4°C. Sections were rinsed several times in PBS and incubated overnight at 4°C with the secondary antibodies. Details on the primary and secondary antibodies used in this study are described in the supplementary information. For immunohistochemistry on slices containing DiI staining, primary antibodies were used in a blocking solution adapted from (Matsubayashi et al., 2008) and containing 3% Normal Goat Serum, 2% DMSO, Digitonin 1mg/ml (Calbiochem). Hoechst (Sigma) was used for fluorescent nuclear counterstaining.

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Slice Culture Experiments

Organotypic slice cultures of the embryonic mouse were prepared as previously described (López-Bendito et al., 2006). Host slices were cut along a 45° section-plane (between sagittal and coronal) which encompass the trajectory of TCA, or along a coronal plane and only intermediate telencephalic slices were selected because they consistently lacked TCA. Brain slices were cultured on polycarbonate culture membranes (8 μm pore size; Whatman) or on PET cell inserts (1 μm pore size; Beckton-Dickinson) in organ tissue dishes containing 1.8 ml of medium BME/HBSS (Invitrogen) supplemented with 20 mM Glucose, 1 mM glutamine, 5% horse serum and 1mM penicilin/streptavidin (Invitrogen). All explants were cut into cubes of approximately 250 μm hedge-length. Cortical explants from *Gfp*-expressing or *tdTomato*-expressing mice (Hadjantonakis et al., 1998; Muzumdar et al., 2007) were grafted at 0 days *in vitro* (DIV) and thalamus explants from wild type or *tdTomato*-expressing mice were grafted at 1 DIV and slices were cultured for 1, 2 or 4 DIV, depending on the experiment.

Collapse assays

Dissociated cultures from E13.5 or E15.5 neocortex and lateral cortex were performed as described (Chauvet et al., 2007). After 24 to 36 hours of culture, neurons were incubated with control or 10 nM AP-Sema3E supernatant for 30 min at 37°C, fixed, immunostained with mouse anti-tubulin antibody (1/2000, Sigma-Aldrich) and labeled with Texas Red-X Phalloidin (1/40, Invitrogen) to analyze growth cone morphologies. Production of mouse AP-Sema3E was performed as described (Chauvet et al., 2007) and collapsed growth cones were scored as in (Castellani et al., 2000).

Image acquisition and analysis

Images were acquired with fluorescence binocular microscope (Leica MZ16 F), fluorescence microscope (Leica DM5000 B) and confocal microscopes (Leica TCS SP2AOBS and TCS SP5). Image analyses were performed with ImageJ, Bitplane Imaris and Adobe Photoshop softwares. The Chi-squared test was used to determine statistical significance of results expressed as percentages of slices or axons.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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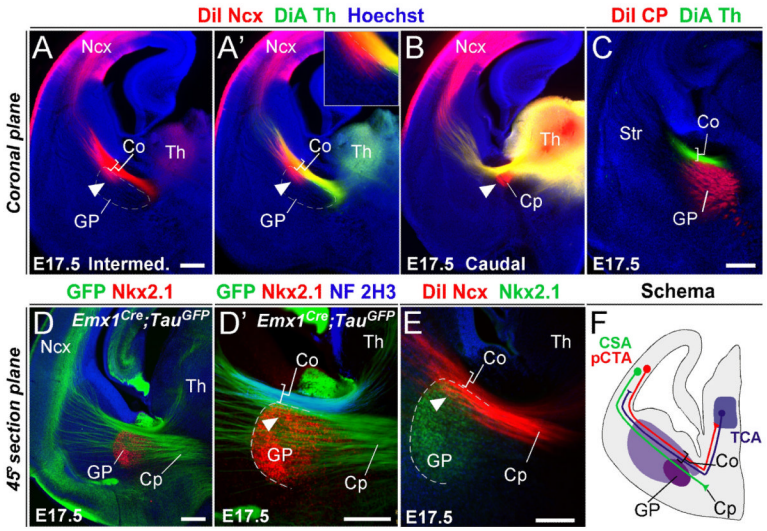

Figure 1. pCTA and CSA follow distinct trajectories in the subpallium

(A-B) Serial coronal sections of a wild type brain at E17.5 after DiI crystal insertions in the somatosensory neocortex and DiA crystal insertions in the thalamus showing distinct pathways for TCA and pCTA on the one hand (A', yellow), and CSA on the other hand (A, A', red, arrowhead). While TCA and pCTA navigate in the corridor, CSA grow in the GP and join the cerebral peduncle (B, arrowhead).

(C) Coronal section of a wild type brain at E17.5 after insertions of DiI and DiA crystals into the cerebral peduncle and thalamus respectively, confirm that CSA navigate through the GP (red) in contrast to TCA and pCTA (green).

(D, D') 45° angle sections (between coronal and sagittal planes) of E17.5 *Emx1^{Cre};Tau^{GFP}* embryos show GFP-positive corticofugal axons passing either through the corridor together with TCA immunostained by neurofilament 2H3 or the globus pallidus (GP) immunostained with Nkx2.1 (arrowhead in D').

(E) 45° angle section at E17.5 showing corticofugal axons labeled by DiI tracing from the neocortex, passing through the corridor and Nkx2.1-positive GP (arrowhead).

(F) Schematic drawing showing the distinct paths followed by TCA (blue), pCTA (red) and CSA (green) within the subpalliums.

Co, corridor; Cp, cerebral peduncle; CSA, corticosubcerebral axons; GP, globus pallidus; Ncx, neocortex; pCTA, pioneer corticothalamic axons; TCA, thalamocortical axons; Th, thalamus.

Scale bars equal 250 μm except 500 μm for A.

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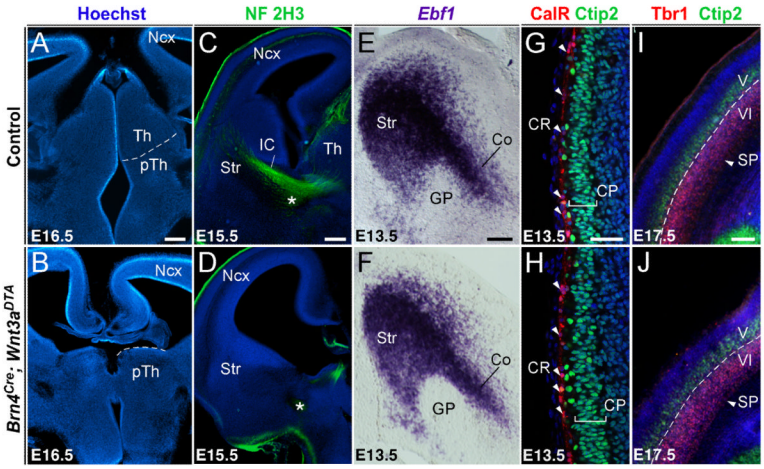

Figure 2. Genetic ablation of the thalamus in *Brn4*^{Cre};*Wnt3a*^{DTA} embryos

(A-B) Hoechst counterstaining of E16.5 coronal sections showing the morphology of a wild type thalamus (A) and its absence in *Brn4*^{Cre};*Wnt3a*^{DTA} embryos (B). Dotted line indicates the boundary between the thalamus and prethalamus.

(C-D) E15.5 coronal sections showing TCA immunostained by Neurofilament 2H3 in control embryos (C) and TCA absence in *Brn4*^{Cre};*Wnt3a*^{DTA} embryos (D). Some low levels 2H3-positive axons are detected superficially to TCA (asterisks).

(E-F) *Ebf1* expression on E13.5 coronal sections is unaffected in the striatum and the corridor of thalamus-ablated embryos (F) compared to the controls (E).

(G-H) E13.5 sections of control (G) and thalamus-ablated (H) embryos showing Calretinin-positive Cajal-Retzius cells (white arrowheads) and Ctip2-positive cortical neurons.

(I-J) Tbr1 and Ctip2 immunohistochemistry at E17.5 showing neocortical layering in control (I) and thalamus-ablated (J) embryos. Dotted lines mark the border between layer V and VI.

Co, corridor; CP, cortical plate; CR, Cajal-Retzius cells; GP, globus pallidus; IC, internal capsule; Ncx, neocortex; pTh, prethalamus; SP, subplate; Str, striatum; Th, thalamus.

Scale bars equal 250 μm (A-D), 100 μm (E, F, I and J) and 50 μm (G, H).

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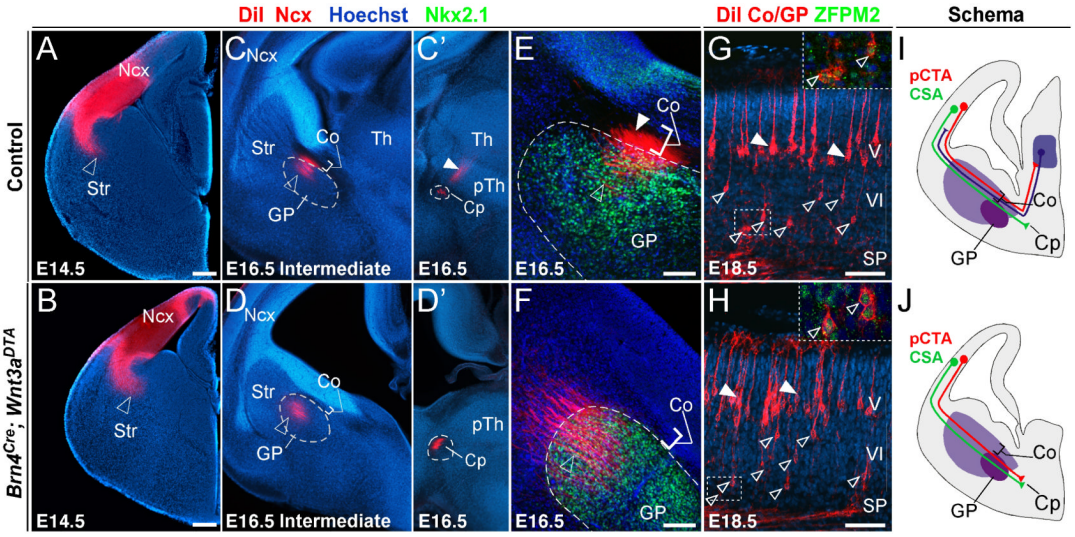

Figure 3. Pathfinding defects of pCTA in the subpallium of thalamus-ablated embryos

(A-B) DiI tracings from the presumptive somatosensory neocortex at E14.5 in control (A) and thalamus-ablated embryos (B) reveal a similar trajectory of corticofugal axons (arrowheads).

(C-F) E16.5 serial coronal sections of a control (C, C') and a thalamus-ablated embryo (D, D') after DiI crystal insertions in the presumptive somatosensory neocortex showing corticofugal axons pathway in the subpallium. In controls, corticofugal axons navigate in the corridor and GP (arrowhead in C) and grow towards the thalamus (solid arrowhead in C') or cerebral peduncle (Cp). In contrast, they all navigate through the GP (arrowhead in D) and into the Cp in mutant embryos (D'). Co-immunostaining with Nkx2.1 (E, F) ascertained the location of axons in the corridor (solid arrowhead) or in the GP (open arrowhead).

(G, H) DiI retrograde labeling from the corridor and GP at E18.5 similarly labeled neurons in layer V (solid arrowhead), layer VI and subplate (open arrowheads) in both control (G) and thalamus-ablated embryos (H). Insets show that back-labelled cells in layer VI are positive for ZFPM2 immunostaining (open arrowheads).

(I, J) Schematic drawing representing the subpallial trajectory of pCTA and CSA in WT (I) and thalamus-ablated (J) embryos.

Co, corridor; Cp, cerebral peduncle; CSA, corticosubcerebral axons; GP, globus pallidus; Ncx, neocortex; pCTA, pioneer corticothalamic axons; pTh, prethalamus; Str, striatum; SP, subplate; Th, thalamus.

Scale bars equal 250 μm (A-D'), 100 μm (E,F) and 50 μm (G,H).

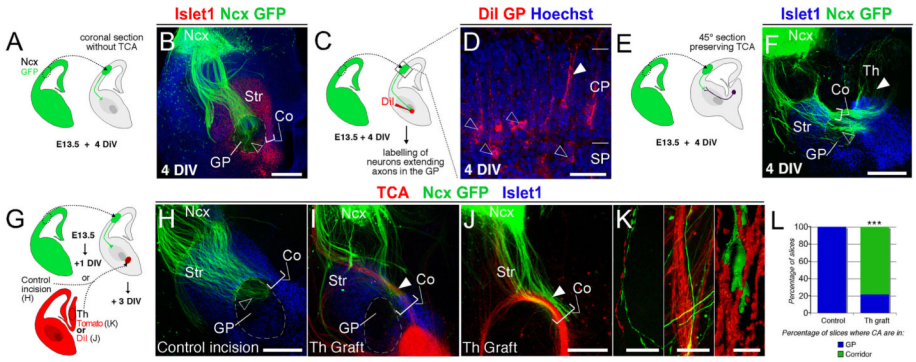

Figure 4. TCA are necessary to guide pCTA in the subpallium

(A) Experimental paradigm to follow *ex vivo* the behavior of corticofugal axons in the absence of TCA.

(B) *Gfp*-expressing cortical explants were homotopically grafted in coronal wild type slices. After 4 days *in vitro* (DIV), corticofugal axons massively invaded the GP (open arrowhead) visualized by lack of Islet1-immunostaining (dotted lines).

(C) Experimental paradigm to test the neuronal origin of axons reaching the GP after 4 DIV.

(D) DiI injection in the GP of slices cultured for 4 DIV back-labeled within the GFP-positive grafted neocortex a vast majority of subplate and deep layer VI neurons that give rise to pCTA (open arrowheads) and very few layer V neurons (solid arrowhead).

(E) Experimental paradigm to follow *ex vivo* the behavior of corticofugal axons into 45° angle slices (between coronal and sagittal planes), which contain the thalamus and TCA.

(F) *Gfp*-expressing cortical explants were homotopically grafted into 45° angle slices (between coronal and sagittal planes) and cultured for 4 DIV. Some corticofugal axons entered the Islet1-negative GP (open arrowhead) and some reached the thalamus (solid arrowhead).

(G) Experimental paradigm to test the role of TCA in pCTA pathfinding.

(H-J) Coronal slices grafted with *Gfp*-expressing cortical explants were cultured for 1 DIV and processed for a control incision at the ventral tip of the corridor (H) or grafts of thalamic explants either expressing *tdTomato* (I) or labeled with DiI (J). After 2 additional DIV, GFP-positive axons entered the GP (open arrowhead) in incised controls, whereas their trajectory was rescued into the Islet1-positive corridor (solid arrowhead) in the presence of TCA.

(K) High magnifications of rescue experiments, showing that axons are close but not in full contact. In the right panel, a 3D reconstruction of axonal surfaces (Bitplane Imaris software) shows an example of physical interactions between TCA and the growth cone of a corticofugal axon.

(L) Quantification of the cases in which pCTA were located in the GP or in the Islet1-positive corridor in TCA-free controls or in the presence of TCA. Chi-squared test, $p<0.0001$.

Co, corridor; CP, cortical plate; DIV, day(s) *in vitro*; GP, globus pallidus; Ncx, neocortex; pCTA, pioneer corticothalamic axons; SP, subplate; Str, striatum; TCA, thalamocortical axons; Th, thalamus.

Scale bars equal 250 μm (B-D, H-J), 50 μm (F,K) except 5 μm for the third panel in K.

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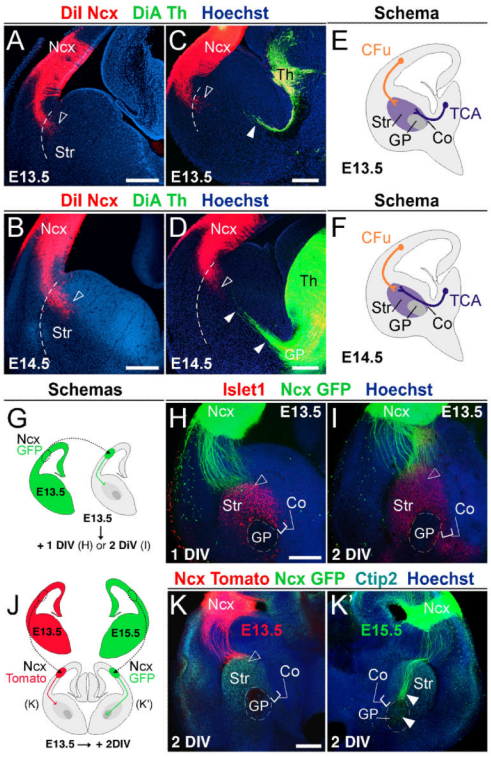

Figure 5. Waiting period of cortical axons coordinates pCTA and TCA progression

(A, B) DiI tracings from the presumptive somatosensory neocortex show that corticofugal axons (CFu) (open arrowheads) stall in the lateral striatum between E13.5 (A) and E14.5 (B). Dotted lines mark the boundary between the cerebral cortex or pallium and the subpallium.

(C, D) DiI/DiA tracings from the presumptive somatosensory cortex and the thalamus in 45° slices at E13.5 (C) and E14.5 (D) indicate that during this time-period, TCA (solid arrowheads) progress up to the pallium/subpallium boundary (dotted lines).

(E, F) Schematic drawings showing the position of CFu at the pallium/subpallial boundary and TCA crossing the subpallium between E13.5 (E) and E14.5 (F).

(G) Experimental paradigm to follow *ex vivo* the behavior of E13.5 corticofugal axons.

(H, I) Grafts of *Gfp*-expressing cortical explants into coronal wild type slices indicate that corticofugal axons (open arrowheads) stall in the Islet1-positive lateral striatum between 1 and 2 days i*n vitro* (DIV).

(J) Experimental paradigm to compare *ex vivo* the behavior of E13.5 and E15.5 corticofugal axons in E13.5 host slices.

(K, K') Homochronic and heterochronic grafts of E13.5 *Tomato*-expressing and E15.5 *Gfp*-expressing cortical explants into an E13.5 host slice show that E13.5 corticofugal axons pause (open arrowhead) whereas E15.5 axons directly progress into the GP (solid arrowheads).

CFu, corticofugal axons; Co, corridor; DIV, day(s) *in vitro*; GP, globus pallidus; Ncx, neocortex; SP, subplate; Str, striatum; TCA, thalamocortical axons; Th, thalamus.

Scale bars equal 250 μm.

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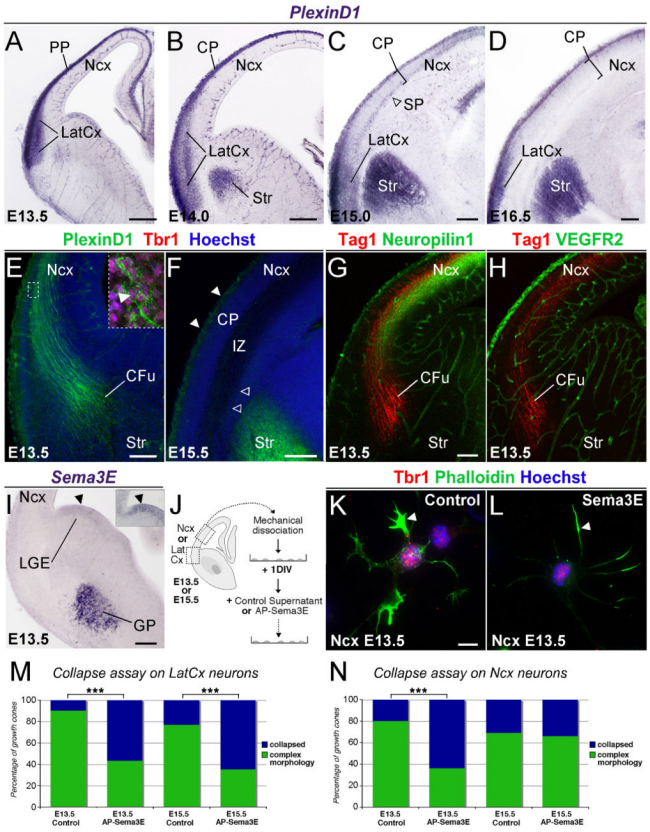

Figure 6. Transient cortical expression of PlexinD1 induces Sema3E-dependant collapse

(A-D) Coronal sections of wild type brains showing transient mRNA expression of *PlexinD1* in the dorsal neocortex preplate at E13.5 (A), cortical plate at E14.0 (B), subplate at E15.0 (arrowhead) (C) and only marginal zone at E16.5 (D). In contrast, *PlexinD1* expression is observed at all stages in the lateral cortex (insular, piriform and perirhinal).

(E, F) PlexinD1 immunostainings on E13.5 and E15.5 coronal sections show that corticofugal axons are transiently labeled. Inset in E indicates that E13.5 Tbr1-positive preplate neurons co-express PlexinD1. At E15.5, PlexinD1 immunolabeling is detected in the marginal zone (solid arrowheads) and striatum but not in corticofugal axons (open arrowheads).

(G-H) E13.5 coronal sections indicating that Tag1-positive corticofugal axons do not co-express Neuropilin1 (G) nor VEGFR2 (H).

(I) E13.5 coronal section showing *Sema3E* expression in the ventricular zone of the LGE (solid arrowhead and inset) and in the GP.

(J) Experimental procedure to test the activity of Sema3E on E13.5 and E15.5 cortical neurons from either the dorsal neocortex or the lateral cortex.

(K-L) Collapse assays on E13.5 dissociated dorsal neocortical neurons identified by Tbr1 immunostaining in the presence of the control supernatant (K) and AP-Sema3E (L). Phalloidin staining shows the complex morphology of growth cones (K) in the control situation or the collapsed morphology (L) in the presence of Sema3E (solid arrowheads).

(M-N) Quantification indicating the percentage of growth cones collapsing in response to control supernatant or AP-Sema3E. Neurons coming from the lateral cortex (M) at E13.5 and E15.5 collapse in the presence of AP-Sema3E whereas neurons from the dorsal neocortex (N) collapse at E13.5 in response to AP-Sema3E and loose their ability to respond at E15.5. Chi-squared test, three asterisks indicate $p<0.0001$ (E13.5 lateral cortex:

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$n_{control}$=209 and 10% of collapsed growth cones / n_{sema3E}=146 and 57% of collapsed growth cones; E15.5 lateral cortex: $n_{control}$=143 and 23% of collapsed growth cones / n_{sema3E}=179 and 65% of collapsed growth cones; E13.5 neocortical neurons: $n_{control}$=168 and 20% of collapsed growth cones / n_{sema3E}=162 and 64% of collapsed growth cones; E15.5 neocortical neurons $n_{control}$=214 and 31% of collapsed growth cones / n_{sema3E}=189 and 34% of collapsed growth cones).

CFu, corticofugal axons; CP, cortical plate; GP, globus pallidus; LatCx, lateral cortex; LGE, lateral ganglionic eminence; Ncx, neocortex; PP, preplate; SP, subplate; Str, striatum.

Scale bars equal 100 μm (A- I) and 10 μm (K,L).

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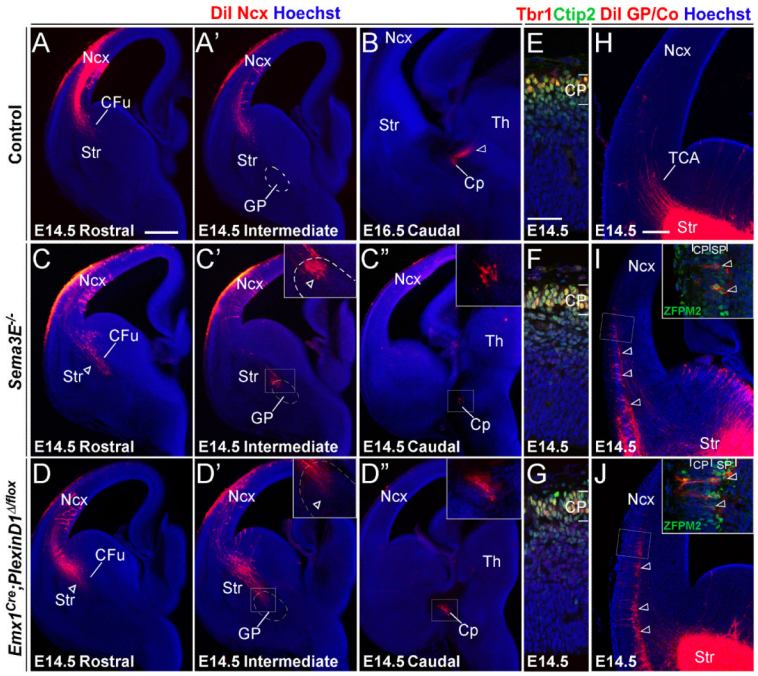

Figure 7. Premature and abnormal growth of pCTA in *Sema3E*$^{-/-}$ and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ embryos

(A-D") Serial coronal sections at E14.5 or E16.5 (B) after DiI crystal insertions in the presumptive somatosensory neocortex of wild type (A,A',B), *Sema3E*$^{-/-}$ (C-C") and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ embryos (D-D"). In wild type brains, corticofugal axons paused at E14.5 and reached the cerebral peduncle (Cp) and thalamus (Th) (open arrowhead in B) at E16.5. However, in E14.5 *Sema3E*$^{-/-}$ and *Emx1*^{Cre}; *PlexinD1*$^{\Delta/flox}$ embryos, corticofugal have crossed the striatum (open arrowheads in C and D) and GP (open arrowheads in the insets in C' and D') and already reached the Cp (insets in C" and D").

(E-G) Double immunochemistry for Tbr1 and Ctip2 on E14.5 coronal sections showing similar co-expression in controls (E), *Sema3E*$^{-/-}$ (F) and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ embryos (G).

(H-J) E14.5 coronal sections showing retrogradely labeled cells in the neocortex after DiI crystal insertions in the globus pallidus and the corridor of wild type (H), *Sema3E*$^{-/-}$ embryos (I) and *Emx1*^{Cre}; *PlexinD1*$^{\Delta/flox}$ embryos (J). Open arrowheads show the presence of back-labeled subplate neurons identified by ZFPM2 co-immunolabeling (insets), only in *Sema3E*$^{-/-}$ (I) and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ embryos (J).

CFu, corticofugal axons; CP, cortical plate; Cp, cerebral peduncle; GP, globus pallidus; Ncx, neocortex; pCTA, pioneer corticothalamic axons; SP, subplate; Str, striatum; TCA, thalamocortical axons; Th, thalamus.

Scale bars equal 500 μm (A-D"), 50 μm (E-G) and 250 μm (I-K).

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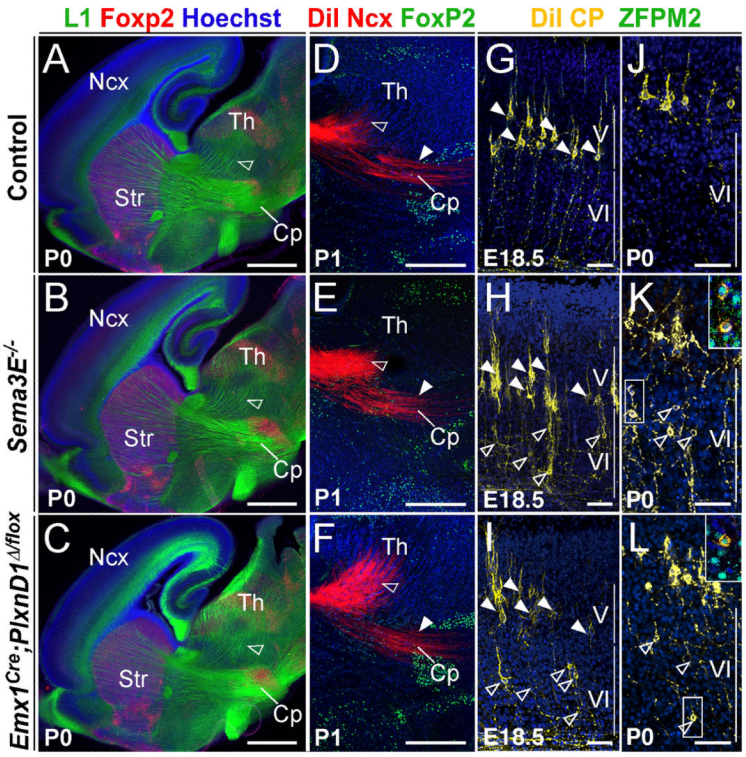

Figure 8. Sema3E/PlexinD1 signaling affects the pathfinding of CTA at birth

(A-C) Sagittal sections of P0 control (A), *Sema3E*$^{-/-}$ (B) and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ (C) mice processed for L1 and FoxP2 immunohistochemistry showing that axons that reached the thalamus (arrowheads) similarly diverged towards their target in contrast to axons that join the cerebral peduncle.

(D-F) Sagittal sections of P1 control (D), *Sema3E*$^{-/-}$ (E) and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ (F) mice after an insertion of a DiI crystal in the somatosensory neocortex showing that in all the genotypes examined, labeled axons that join the cerebral peduncle (solid arrowheads) are segregated from the ones that reach the thalamus (open arrowheads).

(G-L) Coronal sections through the somatosensory neocortex of E18.5 and P0 controls (G,J), *Sema3E*$^{-/-}$ (H,K) and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ (I,L) performed after DiI crystal insertions in the cerebral peduncle at the level of the hypothalamus. In both control and mutant neonates, layer V neurons are back-labeled (solid arrowheads), whereas additional layer VI neurons are stained in both *Sema3E*$^{-/-}$ and *Emx1*^{Cre};*PlexinD1*$^{\Delta/flox}$ mice (open arrowheads). Insets in K and L indicate that layer VI labeled neurons express the CTA marker ZFPM2.

Cp, cerebral peduncle; Ncx, neocortex; Str, striatum; Th, thalamus.

Scale bars equal 500 μm (A-C), 250 μm (D-F) and 50 μm (G-L).